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Bovine NK cells acquire cytotoxic activity and produce IFN-γ after stimulation by *Mycobacterium bovis* BCG- or *Babesia bovis*-exposed splenic dendritic cells

R.G. Bastos [a,b], W.C. Johnson [a], W. Mwangi [c], W.C. Brown [b], W.L. Goff [a,*]

[a] *Animal Disease Research Unit, USDA-ARS, Washington State University, 3003 ADBF, P.O. Box 646630, Pullman, WA 99164-6630, USA*
[b] *Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA*
[c] *Veterinary Pathobiology, Texas A & M University, College Station, TX 77843, USA*

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Abstract

Early interactions of innate immune cell populations, such as dendritic cells (DC) and natural killer (NK) cells, can affect the ability of the acquired immune response to control infection of intracellular microorganisms. In this study, we investigated the activation of bovine NK cells by $CD13^+$ splenic DC stimulated with either *Mycobacterium bovis* BCG or *Babesia bovis* merozoites. Splenic DC were used either immediately after selection (cytokine$^-$) or after exposure to GM-CSF, IL-4 and Flt3L for 72 h (cytokine$^+$). Phenotypic analyses showed up-regulation of MHCII, CD80 and CD86 on cytokine$^+$ DC when compared to cytokine$^-$ DC. Purified NK cells ($CD335^+CD3^-CD2^{+/-}CD8\alpha^{+/-}$) were co-cultured with microbial-exposed cytokine$^-$ DC or cytokine$^+$ DC in either transwell or cell-to-cell format and NK cell IFN-γ production and cytotoxicity were assessed. NK cell IFN-γ production was dependent on cell-to-cell contact. Microbial-stimulated cytokine$^+$ DC induced significantly more IFN-γ production from NK cells than cytokine$^-$ cells. In contrast, cytotoxicity and perforin up-regulation were more pronounced in NK cells cultured with cytokine$^-$ DC than cytokine$^+$ DC. Therefore, activation of bovine NK cells by microbial-stimulated $CD13^+$ splenic DC is influenced by the maturation state of the DC suggesting different roles for the splenic DC during disease-induced maturation.
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Keywords: NK cells; Splenic dendritic cells; Innate immunity; Bovine; Babesiosis; IFN-gamma; Cytotoxicity

1. Introduction

The interaction of accessory cells such as dendritic cells (DC) with natural killer (NK) cells has been described in several studies using human and mouse models (Moretta, 2002; Gerosa et al., 2005; Walzer et al., 2005). Pro-inflammatory cytokine producing accessory cells can prime NK cells for effector and regulatory functions, such as cytotoxic activity and IFN-γ production. In turn, NK cells can induce accessory cell maturation either by direct contact or in synergy with suboptimal levels of cytokines and microbial signals. Thus, interactions between accessory cells and NK cells provide a coordinated mechanism that is involved not only in the regulation of innate immunity, but also in the promotion of appropriate downstream adaptive immune responses (Walzer et al., 2005; Moretta, 2005).

A Th1-biased immune response is an important requirement for the resolution of several infectious diseases in cattle, including tuberculosis and babesiosis

* Corresponding author. Tel.: +1 509 335 6003; fax: +1 509 335 8328.
E-mail address: wgoff@vetmed.wsu.edu (W.L. Goff).

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(Buddle et al., 2005; Brown et al., 2006). In this regard, secretion of IFN-γ is a central component of the response. Although antigen-specific T-cells can produce IFN-γ upon activation, the initial response to pathogen and polarization of the subsequent T-cell response is likely to depend on rapid cytokine secretion by cells of the innate immune system. It is known that, in some cases, secretion of IFN-γ from bovine NK cells is dependent on cytokine production by accessory cells (Goff et al., 2006). Thus, the interaction of accessory cells with NK cells may be required early during the infection to skew the immune response towards a Th1 phenotype and this aspect may be particularly important following infection with intracellular microorganisms, such as *Babesia bovis* and *Mycobacterium bovis*.

Bovine NK cells have been identified and characterized recently as a subset of leukocytes that express the natural cytotoxic receptor NKp46 (CD335), produce IFN-γ and kill target cells (Storset et al., 2004; Goff et al., 2006). Also, $CD13^+$ cells have been recently described as a myeloid DC population present in the spleen that produce inflammatory and regulatory cytokines when exposed to microbial stimulation (Zhuang et al., 2006; Bastos et al., 2007). In the present study, we demonstrate that bovine NK cells acquire cytotoxic activity and produce IFN-γ after stimulation by *M. bovis* BCG- or *B. bovis* merozoite-exposed $CD13^+$ splenic DC. The NK cell IFN-γ production was dependent on cell-to-cell contact and the ability of DC to activate NK cells varied depending on the DC maturation state and type of microbial stimulant. The implications of the DC and NK interactions in modulating the immune response are discussed.

2. Material and methods

2.1. Peripheral blood cells and spleen mononuclear cells

Four Holstein-Friesian steer calves were obtained at 8 weeks of age, vaccinated against pathogenic *Clostridium* species, castrated and dehorned. All animals were cELISA seronegative for *Anaplasma marginale* (VMRD, Pullman, WA, USA) and *B. bovis* (Goff et al., 2003). The calves were maintained according to the American Association for Laboratory Animal Care procedures with acceptable bovine ration, water and mineral block provided *ad libitum*. At 12 weeks of age, each animal underwent a surgical procedure to marsupialize the spleen (Varma and Shatry, 1980). The marsupialized animals were used until 10 months of age as sources of peripheral mononuclear cells (PBMC) and spleen mononuclear cells (SMC) as previously described (Goff et al., 1996).

2.2. Isolation of $CD13^+$ splenic DC and NK cells

SMC were used to obtain $CD13^+$ splenic DC by magnetic positive selection using monoclonal antibody (mAb) CC81 (Table 1) as previously described (Bastos et al., 2007). The expression of CD13, CD172a, CD14, CD80, CD86 and MHC class II (MHCII) on purified splenic DC were assessed using the mAbs described in Table 1 in a FACSCalibur™ workstation (BD Bioscience, Mountain View, CA, USA). For the flow cytometry analyses, mAb CAM36A (anti-CD14) was primarily labeled in house with Alexa Fluor 647 as per manufacture's protocol (Invitrogen) whereas mAbs IL-A159 (anti-CD80) and IL-A190 (anti-CD86) were primarily labeled in house with Alexa Fluor 488 as per manufacture's protocol (Zenon® Mouse IgG Labeling Kit, Invitrogen).

To obtain NK cells, total PBMC were cultivated in Iscove's Medium (Gibco BRL, Gaithersburg. MD, USA) containing 2 μM glutamine, 10 μg/ml gentamicin, 50 μM mercaptoethanol, and 15% fetal bovine serum for 1 week in the presence of 20 ng/ml recombinant human IL-15 (R&D Systems, Minneapolis, MN, USA). After the incubation, the cells were layered onto 20 ml of Hypaque-Ficoll (1.086 g/l, Accupaque, Accurate Chemicals, Westbury, NY, USA) and centrifuged for 15 min at $1500 \times g$ at 4 °C. The interface was collected and washed in 50 ml

Table 1
Monoclonal antibodies

mAb	Isotype	Specificity	Source
CC81	IgG1	CD13	IAH[a]
CC149	IgG2b	CD172a	IAH
AKS1	IgG1	NKp46 (CD335)	Serotec[b]
GB21A	IgG2b	TcR1	VMRD[c]
BAQ44A	IgM	B-cell	VMRD
MM1A	IgG1	CD3	VMRD
CACT138A	IgG1	CD4	VMRD
IL-A159	IgG1	CD80	IAH
IL-A190	IgG1	CD86	IAH
TH14B	IgG2a	MHC class II	VMRD
CAM36A	IgG1	CD14	VMRD
BAQ111A	IgM	CD8α	VMRD
MUC2A	IgG2a	CD2	VMRD

[a] IAH, Institute for Animal Health, Compton, England, United Kingdom (provided by Chris Howard).
[b] Serotec, Raleigh, NC, USA.
[c] VMRD, Veterinary Medical Research and Development, Pullman, WA, USA.

of Dulbecco's Modified Eagle's Medium (DMEM, pH 7.35). The cells were counted and depleted of monocytes, γδ T lymphocytes, B lymphocytes and αβ T lymphocytes. For depletion, the MagCellect system (R&D Systems, Minneapolis, MN, USA) was used following the manufacture's instructions. Briefly, 5×10^7 IL-15-incubated PBMC were washed once in phosphate buffered saline supplemented with 0.5% BSA 2 mM EDTA (PBS-BE) and incubated for 15 min at 4 °C with 6 μg of mAbs CC149, BAQ44A, CACT138A, MM1A and GB21A (Table 1). After incubation, the cells were washed twice in PBS-BE and incubated for 15 min at 4 °C with 20 μl of goat anti-mouse IgG + IgM biotin conjugate (Caltag Laboratories, Burlingame, CA, USA). The cells were washed twice as described before and incubated for 15 min at 4 °C with 150 μl of MagCellect Streptavidin Ferrofluid (R&D Systems). Labeled cells were retained using a MagCellect magnet, the unlabeled cells were collected and the expression of CD335, CD3, CD2 and CD8α surface markers was assessed by flow cytometry using a FACSCalibur™ workstation (BD Bioscience). For the flow cytometry analyses, mAb MM1A (anti-CD3) was primarily labeled in house with Alexa Fluor 647 as per manufacture's protocol (Invitrogen).

2.3. *Babesia bovis merozoites, Mycobacterium bovis BCG and cytokines*

Viable *B. bovis* merozoites (Mz) were isolated by Percoll gradient as previously described (Goff et al., 1988) and used at a ratio of 10 merozoites per leukocyte. *M. bovis* BCG, sub-strain Pasteur, was grown for 2 weeks without shaking in Middlebrook 7H9 liquid medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10% albumin–dextrose complex (ADC) enrichment, 0.01% cyclohexamide and 0.05% Tween 80 as previously described (Bastos et al., 2002). It has been demonstrated in vivo that heat-killed BCG (HK-BCG) can prime and modulate the immune system (Shibata et al., 2001; Yamashita et al., 2007). In addition, we demonstrated that in vitro HK-BCG is able to induce expression of inflammatory cytokines from monocytes and DC (Bastos et al., 2007). Therefore, the bacteria were washed, air-dried, suspended in sterile PBS at 1 mg/ml and heated at 95 °C for 30 min. HK-BCG were used at 50 μg/ml as previously described (Bastos et al., 2007).

Recombinant bovine GM-CSF and recombinant bovine Flt3L were produced as previously described (Mwangi et al., 2002; Zhuang et al., 2006) and used when indicated at 2 ng/ml and 100 ng/ml, respectively. Recombinant bovine IL-4 (R&D Systems), recombinant human IL-12 (R&D Systems) and recombinant human IL-18 (Medical & Biological Labs, Woburn, MA, USA) were used when indicated at 1 ng/ml, 20 ng/ml and 0.2 ng/ml, respectively as previously described (Goff et al., 2006; Bastos et al., 2007).

2.4. *Co-culture of splenic DC and NK cells*

For NK cell activation, positively selected $CD13^+$ splenic DC were either used immediately after the selection (cytokine$^-$) or after exposure to GM-CSF, IL-4 and Flt3L for 72 h (cytokine$^+$). Cytokine$^-$ or cytokine$^+$ splenic DC (5×10^5 cells/ml) were incubated for 24 h with either HK-BCG or Mz and then NK cells (10^6 cells/ml) were added and co-cultured for 24 h. The co-cultures were performed in either transwell or cell-to-cell contact formats. For transwell cultures, two-chamber 24-well plates with 0.4 μm pore (Costar, Cambridge, MA, USA) were used. After the incubation, the cells were collected for RT-PCR and cytotoxic assay and the supernatants checked for the presence of IFN-γ by ELISA (Prionics USA, Inc., Lincoln, NE, USA) per the manufacture's instructions. The IFN-γ results were calculated based on a standard curve ranging from 1.56 U/ml to 100 U/ml. The means of IFN-γ production within treatments were compared using one-way analysis of variance followed by Tukey's W test.

2.5. *mRNA expression of bovine perforin*

Cellular RNA was isolated using 1 ml TRIzol (BRL, Bethesda, MD, USA) containing 20 μg glycogen (Boehringer Mannheim, Germany). RNA samples were used for RT-PCR amplification of bovine perforin using forward 5′-gatgccaacttcgccgccca-3′ and reverse 5′-tgtcagtcacgtacttgctc-3′ primers. The PCR condition was 30 cycles of 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. The transcript level for each sample was estimated by densitometry image analysis, where the GAPDH housekeeping gene measurement was used as the standard (IS-1000 Digital Imaging System and Alpha-EASE 3.21 software program, San Francisco, San Leandro, CA, USA). Progressive 10-fold dilutions of GAPDH cDNA were evaluated and optimized by densitometry to confirm the reliability of the method as a semi-quantitative measure of mRNA expression (correlation coefficient of dilution versus densitometric values: $r = 0.966$). The mRNA level of bovine perforin is presented as relative units after normalization to the observed GAPDH level.

2.6. Cytotoxic assay

NK cells were co-cultured with and without microbial-stimulated splenic DC and the cytotoxic activity of NK cells against MDBK (ATCC, Rockville, MD, USA) target cells was evaluated using the flow cytometry-based PKH-26 assay as previously described (Fischer and Mackensen, 2003). Briefly, monolayers of MDBK cells were grown in DMEM with 15% fetal bovine serum, washed twice in serum free-medium and detached using trypsin. MDBK cells (2×10^7 cells/ml) were labeled using PKH-26 Red Fluorescent Cell Linker Kit (Sigma, Mo, USA) as per the manufacture's instructions. Effector NK cells and MDBK PKH-26 labeled target cells were incubated at 0.5:1, 1:1, 5:1, and 10:1 *E/T* ratios in a 96-well-V-bottom plate for 3 h at 37 °C 5% CO_2. After incubation, the cells were harvested, washed twice in PBS and suspended in 100 μl of binding buffer (Hepes 2.303 g/l, NaCl 8.182 g/l, $CaCl{\cdot}2H_2O$ 0.368 g/l, pH 7.4). The cells were then stained with 5 μl of Annexin V-FITC (BD Pharmingen, USA) for 10 min at room temperature in the dark and analyzed using a FACSCalibur™ (Becton Dickinson, San Jose, CA, USA). The cytotoxicity was calculated by the percentage of PKH-26 labeled cells that acquired Annexin V stain. In a blocking experiment, NK cells (10^6 cells/ml) were preincubated with 1 μg/ml mAb AKS1 for 30 min and then incubated with target cells as described above. mAb MM1A (Table 1) was used in the blocking experiments as a negative isotype control. The means of cytotoxicity were compared using one-way analysis of variance followed by Tukey's W test.

3. Results

3.1. Purification and characterization of splenic DC and NK cells

In a previous report we described a $CD13^+$ $CD11a^-CD11b^{+/-}CD11c^+CD14^-CD172a^{+/-}MHCII^+$ $CD205^+$ myeloid splenic DC population (Bastos et al., 2007). Here we analyzed the phenotype of this population immediately after the CD13 positive selection (cytokine$^-$ DC) (Fig. 1A) and after exposure to GM-CSF, IL-4 and Flt3L for 72 h (cytokine$^+$ DC) (Fig. 1B). The data showed that CD13 expression was down-regulated on cytokine$^+$ DC and approximately 15% of the purified cytokine$^-$ DC co-expressed CD172a which was up-regulated to more than 50% on cytokine$^+$ DC (Fig. 1, panels A and B). Moreover, both cytokine$^-$ and cytokine$^+$ DC failed to express the monocyte marker CD14 (Fig. 1, panel C). Up-regulation of MHCII, CD80 and CD86 was also apparent on cytokine$^+$ DC with increased expression of 10%, 35% and 30%, respectively (Fig. 1 panels A, B and C).

Bovine NK cells from PBMC were previously demonstrated to expand in culture in the presence of recombinant human IL-15 (Goff et al., 2006). Here we further purified a NK cell population from such cultures by depletion of B-cells, T-cells (including γδ T-cells) and monocytes. The depletion method was used as opposed to positive selection in order to avoid possible blocking of cell cytotoxicity and/or triggering of cell pathways by antibody binding to the specific cell receptor. The purified NK cell population was characterized as $CD335^+$, $CD3^-$, $CD2^{+/-}$ and $CD8\alpha^{+/-}$ (Fig. 2). Two-color FACS analyses showed that only 15% of the purified NK cell population co-express CD2 and CD8α markers (Fig. 2).

3.2. IFN-γ production by NK cells

Numerous reports demonstrate that IFN-γ production by NK cells requires IL-12 induction that is enhanced in the presence of IL-18. Both cytokines can be produced by DC and monocytes/macrophages after microbial activation. We examined the ability of splenic DC exposed to either HK-BCG or Mz to induce IFN-γ production from NK cells in the presence or absence of exogenous IL-18 and whether the induction required cell-to-cell contact.

In a transwell configuration, both cytokine$^-$ and cytokine$^+$ DC failed to induce IFN-γ production from NK cells with or without microbial activation (Table 2). In addition, NK cells co-cultured with non-activated DC (Table 2) or exposed directly to either HK-BCG or Mz (data not shown) did not produce IFN-γ suggesting that the presence of the microbial-activated DC was necessary for the NK cell IFN-γ production.

Using HK-BCG for microbial activation in the cell-to-cell contact format, cytokine$^+$ DC were able to prime NK cells to produce IFN-γ. Moreover, the addition of a suboptimal concentration of IL-18 to co-cultures of HK-BCG-exposed cytokine$^+$ DC and NK cells increased more than 15 times the NK cell IFN-γ production. The suboptimal concentration of IL-18 alone had no effect (data not shown). Interestingly, cytokine$^-$ DC exposed to HK-BCG failed to induce production of IFN-γ by NK cells demonstrating that, in the presence of mycobacterial antigens, some level of DC maturation is required to prime NK cells to produce IFN-γ (Table 2).

Cytokine$^-$ DC exposed to Mz, unlike HK-BCG, induced significant ($P < 0.05$) NK cell IFN-γ production in cell-to-cell contact cultures. However, the

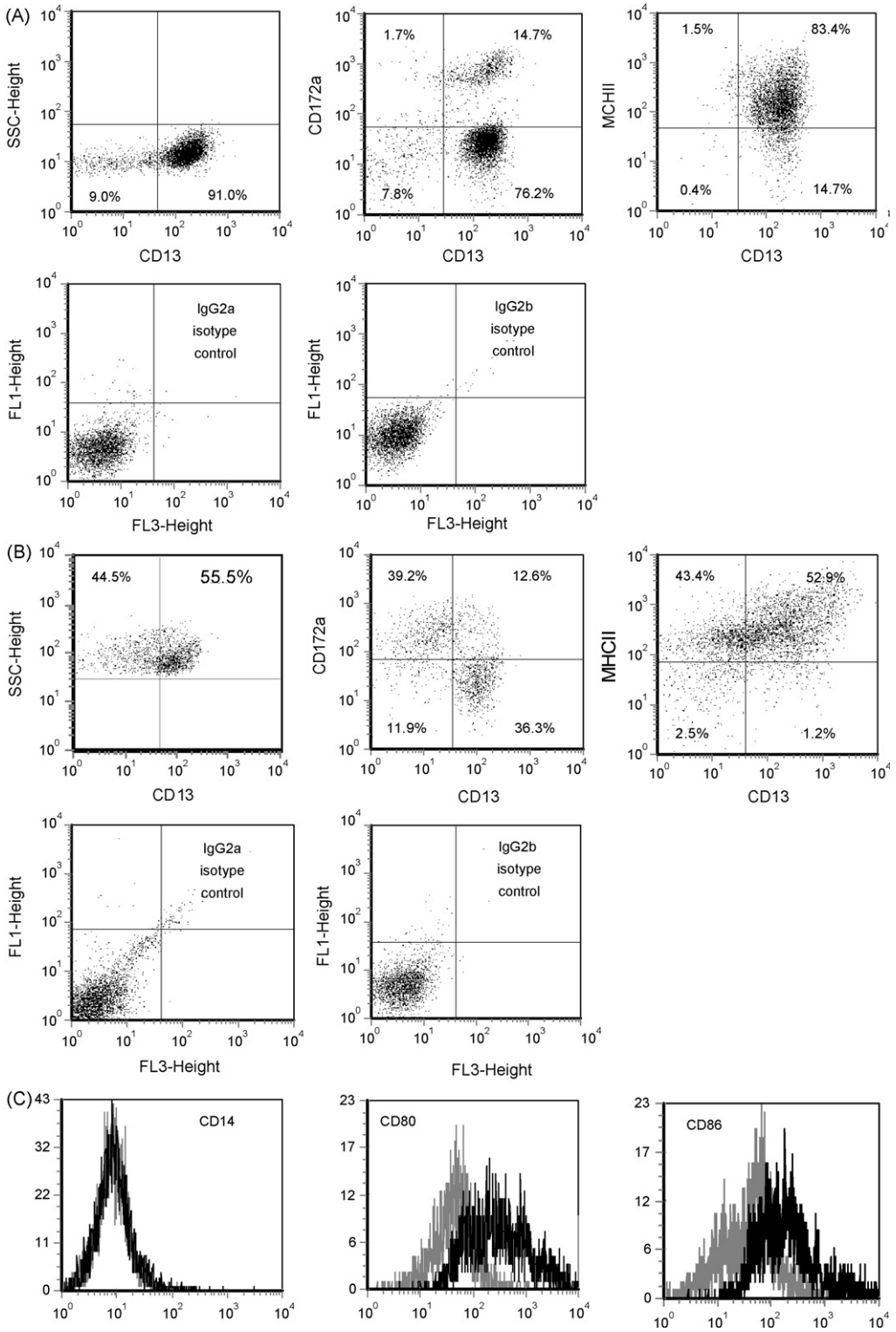

Fig. 1. Flow cytometry analysis to investigate the expression of CD13, CD172a, MHC class II, CD80, CD86 and CD14 on the surface of splenic DC. Positively selected CD13^{+} splenic DC were phenotypically analyzed either immediately after the selection (cytokine^{-} DC) (panel A) or after exposure to GM-CSF, IL-4 and Flt3L for 72 h (cytokine^{+} DC) (panel B). The histograms presented in panel C show the expression of CD14, CD80 and CD86 on cytokine^{-} DC (grey line) and cytokine^{+} DC (black line). mAb CAM36A (anti-CD14) was primarily labeled in house with Alexa Fluor

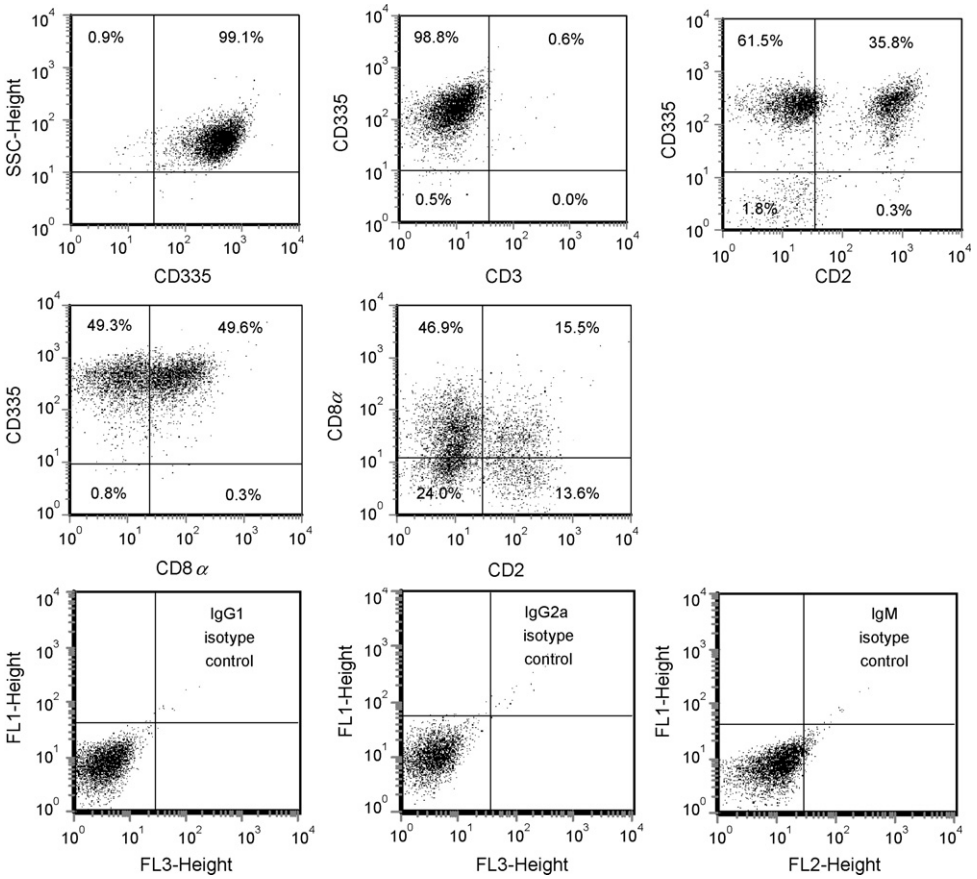

Fig. 2. Phenotype of purified NK cells. NK cell population was obtained by incubating total PBMC with recombinant human IL-15 for 1 week followed by depletion of B lymphocytes, αβ-T lymphocytes, γδ-T lymphocytes and monocytes. Purified NK cells were identified as $CD335^{+}$, $CD3^{-}$, $CD2^{+/-}$ and $CD8^{+/-}$. The mAb MM1A (anti-CD3) was primarily labeled in house with Alexa Fluor 647 as per manufacture's protocol (Invitrogen). The data are representative of four calves used in this study.

addition of IL-18 did not have an effect in increasing the IFN-γ production. On the other hand, $cytokine^{+}$ DC exposed to Mz induced nearly five times more NK cell IFN-γ production than $cytokine^{-}$ DC and the addition of suboptimal concentration of IL-18 resulted in two-fold increase in the IFN-γ production (Table 2).

3.3. Cytotoxic activity of NK cells

Several studies suggest that IFN-γ production and cytotoxicity are associated with NK cells. Therefore, we evaluated the cytotoxic activity of purified NK cells co-cultured with microbial-activated DC. The flow cytometry-based PKH-26 assay was used to evaluate NK cell cytotoxicity. For this assay, MDBK target cells were labeled with PKH-26 dye (Fig. 3, panel A, dot plot 1), incubated with the effector NK cells at different *E/T* ratios and the cytotoxicity was calculated by the percentage of labeled target cells that acquired Annexin V-FITC stain. Fig. 3, panel A, dot plot 2 shows the percentage of MDBK PKH-26 labeled target cells exposed to saponin for 30 min and stained with Annexin V-FITC. NK cells alone exposed to HK-BCG or Mz lysed $\leq$10% target cells (*E/T* ratio of 10:1) and that was considered the baseline for the assay (Fig. 3, panel A, dot plots 3 and 4). In addition, $\leq$10% of target cells were lysed in the presence of either $cytokine^{-}$ or $cytokine^{+}$ DC alone (DC/target cell ratio of 10:1) (Fig. 3, panel A, dot plots 5 and 6). Fig. 3, panel B, shows that NK cells co-cultured with microbial-exposed $cytokine^{-}$ or $cytokine^{+}$ DC efficiently lysed the MDBK target cells. Interestingly, the NK cell

647 as per manufacture's protocol (Invitrogen). mAbs IL-A159 (anti-CD80) and IL-A190 (anti-CD86) were primarily labeled in house with Alexa Fluor 488 as per manufacture's protocol (Zenon® Mouse IgG Labeling Kit, Invitrogen). The data are representative of four calves used in this study.

Table 2
Production of IFN-γ by NK cells co-cultured with splenic DC stimulated with either heated-killed *M. bovis* BCG or *B. bovis* merozoites

Treatment	Transwell		Cell-to-cell	
	Cytokine$^-$ DC[a]	Cytokine$^+$ DC[b]	Cytokine$^-$ DC	Cytokine$^+$ DC
DC + NK	0.59 (0.211)	0.18 (0.093)	1.01[a] (0.077)	0.58[a] (0.066)
DC + HK-BCG	ND	ND	0.04[a] (0.061)	0.18[a] (0.107)
DC + HK-BCG + NK	0.26 (0.158)	0.02 (0.025)	0.85[a] (0.153)	6.20[b] (0.263)
DC + HK-BCG + IL-18 + NK	0.08 (0.006)	0.09 (0.018)	0.25[a] (0.015)	106.72[c] (0.771)
DC + NK	0.59 (0.211)	0.18 (0.093)	1.01[a] (0.077)	0.58[a] (0.066)
DC + Mz	ND	ND	0.08[a] (0.011)	0.08[a] (0.013)
DC + Mz + NK	0.12 (0.030)	0.09 (0.020)	10.39[b] (2.539)	48.22[c] (0.057)
DC + Mz + IL-18 + NK	0.10 (0.011)	0.11 (0.004)	10.91[b] (2.273)	96.94[d] (3.555)

[a] Positively selected splenic DC were stimulated with either heat-killed *M. bovis* BCG (HK-BCG) or *B. bovis* merozoites (Mz) and co-cultured with NK cells.

[b] Positively selected splenic DC were exposed to IL-4, GM-CSF, and Flt3L, as described in Section 2, stimulated with either HK-BCG or Mz and co-cultured with NK cells. Means of IFN-γ production are shown as U/ml and standard deviations are shown in parenthesis. ND—not done. Data are representative of four calves used in this study. Means, within microbial treatment, followed by different letters are significantly different ($P < 0.05$) by Tukey's W test.

cytotoxicity was significantly increased ($P < 0.05$) when co-cultured with microbial-exposed cytokine$^-$ DC than with cytokine$^+$ DC. Moreover, preincubation with mAb AKS1 reduced significantly ($P < 0.05$) the NK cell cytoxicity, demonstrating the participation of CD335 in the cytotoxic activity.

Perforin mRNA expression from NK cells was up-regulated in co-cultures with microbial-exposed DC (Fig. 4). The up-regulation of NK cell perforin was most pronounced in co-cultures with microbial-exposed cytokine$^-$ DC. In fact, perforin message was not detectable from the Mz-activated cytokine$^+$ DC condition although cytotoxicity was apparent, suggesting that this secretory mechanism may not be the operative cytotoxic mechanism under this condition.

4. Discussion

The development of a mAb to bovine NKp46 (CD335) (Storset et al., 2004) has allowed characterization of a bovine NK cell population demonstrating both effector and regulatory functions that can affect downstream acquired immune responses (Olsen et al., 2005; Goff et al., 2006; Boysen et al., 2006a, 2006b; Klevar et al., 2007). In this paper we show that bovine NK cells acquire cytotoxic activity and produce IFN-γ after in vitro stimulation by either HK-BCG- or Mz-exposed splenic DC.

The bovine NK cell population used in this study was characterized as CD335$^+$CD3$^-$CD2$^{+/-}$ and CD8α$^{+/-}$. The NK cell phenotype was consistent in all four calves used in this study and is in agreement with previous reports (Storset et al., 2004; Goff et al., 2006).

CD13$^+$ DC have been previously described as a bovine myeloid DC population present in the spleen and afferent lymph, but not in peripheral blood, that produce inflammatory cytokines and stimulate an antigen-specific recall response (Howard et al., 1997; Zhuang et al., 2006; Bastos et al., 2007). We previously demonstrated that approximately 15% of positively selected splenic CD13$^+$ DC expressed CD172a (Bastos et al., 2007). Here we confirm this result and show that CD172a is up-regulated on cytokine$^+$ DC. However, the biological relevance of the up-regulation of CD172a on these cells remains to be elucidated. Additionally, we demonstrate a down-regulation of CD13 expression on cytokine$^+$ DC. Assuming that incubation with GM-CSF, IL-4 and Flt3L induced some level of maturation in the DC population, the data here confirm previous studies suggesting that CD13 may be a marker for immature splenic DC (Bendriss-Vermare et al., 2001; Zhuang et al., 2006; Bastos et al., 2007). Moreover, MHCII, CD80 and CD86 were up-regulated on cytokine$^+$ DC when compared to cytokine$^-$ cells indicating that incubation with GM-CSF, IL-4 and Flt3L induces some level of DC maturation as previous reported (Zhuang et al., 2006).

IFN-γ is an important component in the induction of a type-1 immune response for controlling intracellular microorganisms. It has been proposed that early production of IFN-γ is crucial to control *B. bovis* infection (Brown et al., 2006). In addition, it was recently reported that bovine NK cells act as early IFN-γ producers in *Neospora caninum* infection skewing the immune response towards a type-1 response (Klevar et al., 2007) and that NK cells could be activated directly with this parasite (Boysen et al., 2006a, 2006b). The

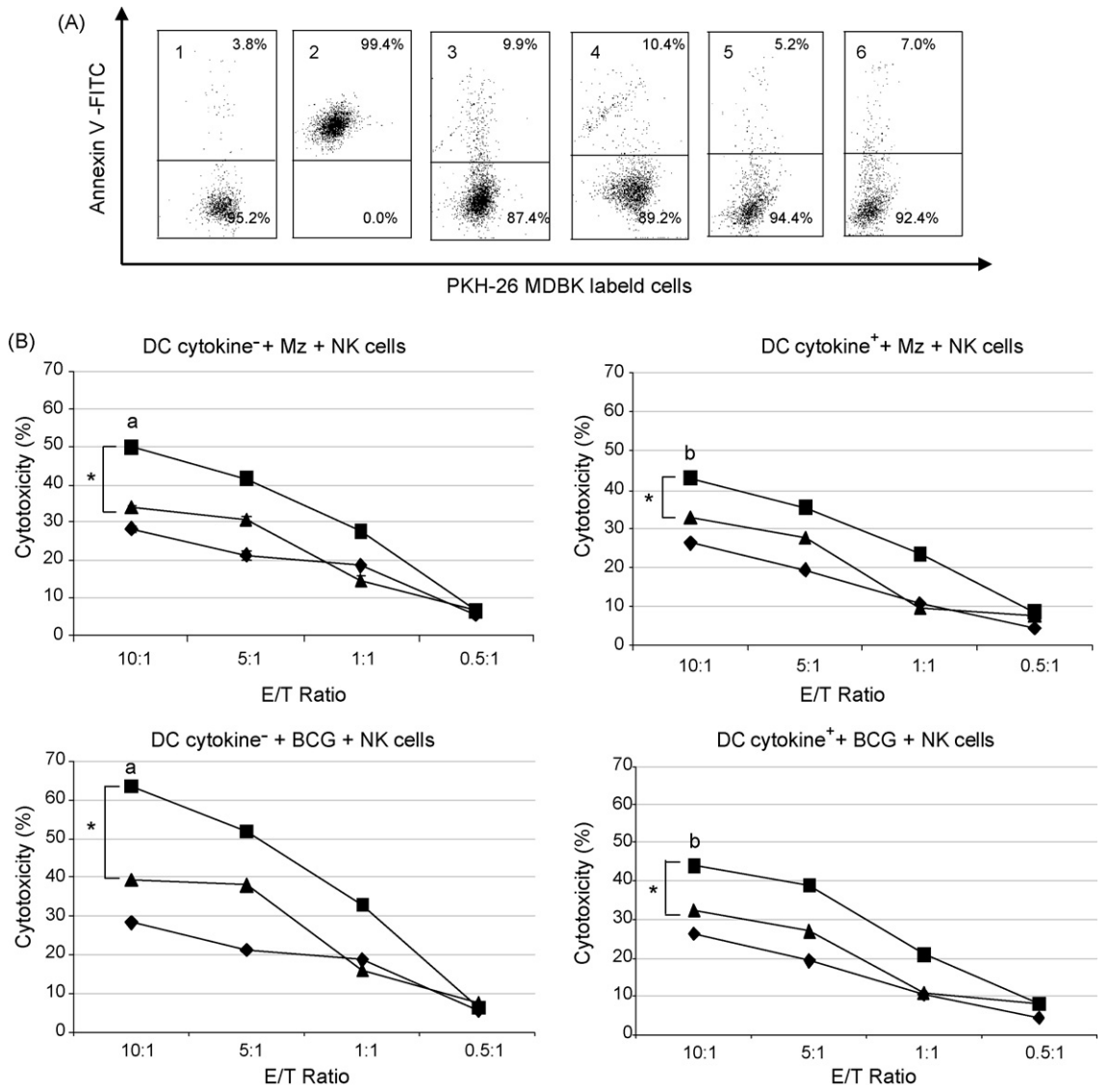

Fig. 3. Cytotoxic activity of NK cells co-cultured with either cytokine$^-$ or cytokine$^+$ splenic DC activated with either heat-killed *Mycobacterium bovis* BCG (HK-BCG) or *Babesia bovis* merozoites (Mz). Panel A dot plots 1 and 2 show MDBK PKH-26 labeled target cells before and after incubation with saponin, respectively. Dot plots 3 and 4 show target cells in the presence of Mz- or HK-BCG-exposed NK cells alone (*E/T* ratio of 10:1), respectively. Dot plots 5 and 6 show target cells in the presence of either cytokine$^-$ or cytokine$^+$ splenic DC alone (DC/target cell ratio of 10:1), respectively. Panel B shows the cytotoxic activity of NK cells co-cultured with microbial-exposed cytokine$^-$ or cytokine$^+$ DC. (◆) DC plus NK cells. (■) Microbial-exposed DC plus NK cells. (▲) Microbial-exposed DC plus NK cells preincubated with mAb AKS1. Means, within microbial treatment, followed by different letters are significantly different ($P < 0.05$) by Tukey's W test. Asterisks indicate significant differences ($P < 0.05$) by Tukey's W test between means within the same *E/T* ratio. The data are representative of four calves used in this study.

results of the current study demonstrate the complexity of the NK cell IFN-γ production involving the influence of different microbial agents, maturation state of the accessory cells and the need for accessory/effector cell contact. All aspects have been noted in similar cell interactions from other species (Moretta et al., 2003; Lanier, 2005). It has been shown that bovine NK cells are able to produce IFN-γ in response to cytokines and antigen-exposed monocytes/macrophages (Storset et al., 2004; Olsen et al., 2005; Goff et al., 2006; Endsley et al., 2006; Denis et al., 2007). It has also been demonstrated that human DC are incapable of triggering resting NK cells without a microbial stimulus (Fernandez et al., 2002). Our results are in agreement with these observations where only microbial-exposed cytokine$^+$ DC are able to induce IFN-γ production from NK cells.

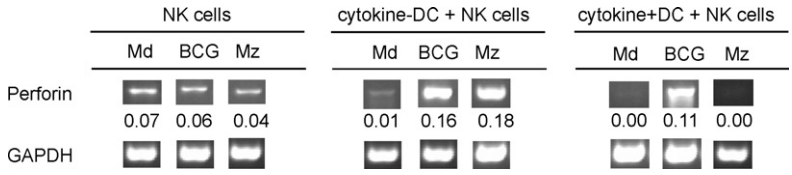

Fig. 4. Perforin RNA expression from NK cells co-cultured with heat-killed *M. bovis* BCG (HK-BCG)- or *B. bovis merozoites* (Mz)-exposed splenic DC. Md indicates co-cultures without microbial stimulation. The transcript level for each sample was estimated by densitometry image analysis, where the GAPDH measurement was used as the standard. The mRNA level of bovine perforin is presented as relative units after normalization to the observed GAPDH level. The data are representative of four calves used in this study.

It has been shown that monocytes induce IFN-γ production by NK cells without the need for cell-to-cell contact (Olsen et al., 2005). However in the current study, cell-to-cell engagement was necessary for splenic DC priming of NK cells to produce IFN-γ. This is in agreement with other reports in humans describing the requirement of cell-to-cell contact for NK cell activation by DC (Piccioli et al., 2002; Gerosa et al., 2002; Ferlazzo et al., 2002; Borg et al., 2004). Additionally, in the cell-to-cell contact format, Mz-activated cytokine$^+$ DC triggered a greater ($P < 0.05$) NK cell IFN-γ production than HK-BCG-activated cells suggesting that qualitative and quantitative differences between protozoal and bacterial PAMPs, and their engagement with bovine DC-associated TLRs, can also have profound differences in the NK cell IFN-γ production.

Although there have been numerous reports suggesting that GM-CSF and IL-4 with or without Flt3L can induce some level of maturation in DC, these cells are still considered immature DC until either TNF-α and/or microbial/antigen stimulation has occurred (Iwamoto et al., 2007; Wang et al., 2007). We provide evidence that GM-CSF, IL-4 and Flt3L have an effect in the ability of DC to prime NK cells particularly in increasing IFN-γ production. Further studies are necessary to better define the maturation status of bovine splenic DC under culture conditions.

Microbial exposure can lead to production of only IL-12 from some DC populations while inducing other DC and monocyte/macrophages to produce both IL-12 and IL-10 (Hope et al., 2004; Bastos et al., 2007). IL-10 has been shown to be restricted to CD14$^+$ cells (de Saint-Vis et al., 1998; Hope et al., 2004; Bastos et al., 2007) and its induction appears to be dominant, restricting IL-12 production from the same cell and other cells to low levels (Haase et al., 2002). We previously demonstrated that NK cells proliferate in vitro in the presence of IL-15 but required IL-12 and IL-18 for production of IFN-γ. Moreover, IL-12 accumulated in supernatants of Mz-exposed monocytes induced NK cell IFN-γ production when a suboptimal concentration of IL-18 was added (Goff et al., 2006). It was beyond the scope of this study to determine the cytokines involved in the NK cell activation by DC. However, since the addition of a suboptimal concentration of IL-18 augmented IFN-γ levels significantly ($P < 0.05$) when microbial-activated DC were cultured in cell-to-cell contact with NK cells, one may speculate that IL-12 is involved in a synergistic way with IL-18 to increase the IFN-γ response (Goff et al., 2006). Moreover, a role for IL-15 cannot be ruled out since the trans-presentation of IL-15 by DC to NK cells has been reported recently as a requirement for proliferation and priming of NK cells (Sandau et al., 2004; Lucas et al., 2007).

Apart from their role as regulatory cells, NK cells are well known for their ability to lyse virally infected target cells (Lanier, 2005). However, the physiological significance of NK cell cytotoxicity in bacterial and protozoal infections is still a matter of debate (Scharton-Kersten and Sher, 1997; Korbel et al., 2004). Cytotoxic activity by bovine NK cells has been previously reported by others (Storset et al., 2004; Endsley et al., 2006) and here we demonstrate both cytotoxicity and perforin up-regulation by bovine NK cells co-cultured in cell-to-cell contact format with microbial-activated splenic DC. Interestingly, the NK cell cytotoxicity and perforin expression were more pronounced when co-cultured with cytokine$^-$ rather than cytokine$^+$ splenic DC and perforin message was not detected at all from the Mz-activated cytokine$^+$ DC condition. However, the MDBK target cells were lysed by NK cells from the Mz-activated cytokine$^+$ DC cultures suggesting the involvement of another lytic mechanism. Two constitutive cytotoxic mechanisms have been attributed to NK cells; a secretory/necrotic mechanism involving perforin and an apoptotic mechanism involving NK cell-associated Fas ligand interaction with Fas bearing target cells (Oshimi et al., 1996). In addition, IL-4 has been shown to down-regulate perforin-mediated cytotoxicity while enhancing Fas ligand-mediated cytotoxicity (Aung and Graham, 2000). Therefore, it is possible that the protozoal activation along with DC cultured in the presence of exogenously added IL-4,

inhibits the perforin-mediated cytotoxicity while promoting Fas ligand-mediated killing of the Fas-receptor bearing MDBK target cells. Both the NK cell secretory and apoptotic cytolytic mechanisms are enhanced by cytokines such as IL-2 or IL-15 together with IL-12 (Medvedev et al., 1997; Aung and Graham, 2000; Endsley et al., 2006). The NK cells used in the current study were cultured in the presence of IL-15 and the microbial-activated $CD13^+$ splenic DC have been shown to up-regulate IL-12 (Bastos et al., 2007) suggesting a role for both cytokines in the NK cell activation. In addition, MDBK target cell lysis was significantly reduced ($P < 0.05$) by preincubating NK cells with mAb AKS1, indicating that CD335 is necessary but not sufficient for cytotoxicity as previously reported (Storset et al., 2004).

In conclusion, we show that bovine NK cells can respond to microbial-exposed splenic DC with both regulatory and effector activities. The results demonstrate the influence of accessory DC phenotype and maturation state on the ability to induce NK cell activity along with the importance of the microbial agent being processed. In addition, we demonstrate that NK cell activation by DC is cell-to-cell contact-dependent. These data are supported by immunohistologic evidence for DC/NK cell contact during the early response to *B. bovis* infection (manuscript in preparation). Further studies are necessary to evaluate the reciprocal interaction between NK cells and DC and determine the relevance of the DC maturation stage in the cross-talking with bovine NK cells.

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References

Aung, S., Graham, B.S., 2000. IL-4 diminishes perforin-mediated and increases Fas ligand-mediated cytotoxicity in vivo. J. Immunol. 164, 3487–3493.

Bastos, R.G., Dellagostin, O.A., Barletta, R.G., Doster, A.R., Nelson, E., Osorio, F.A., 2002. Construction and immunogenicity of recombinant *Mycobacterium bovis* BCG expressing GP5 and M protein of porcine reproductive respiratory syndrome virus. Vaccine 21, 21–29.

Bastos, R.G., Johnson, W.C., Brown, W.C., Goff, W.L., 2007. Differential response of splenic monocytes and DC from cattle to microbial stimulation with *Mycobacterium bovis* BCG and *Babesia bovis* merozoites. Vet. Immunol. Immunopathol. 115, 334–345.

Bendriss-Vermare, N., Barthelemy, C., Durand, I., Bruand, C., zutter-Dambuyant, C., Moulian, N., Berrih-Aknin, S., Caux, C., Trinchieri, G., Briere, F., 2001. Human thymus contains IFN-alpha-producing CD11c(−), myeloid CD11c(+), and mature interdigitating dendritic cells. J. Clin. Invest. 107, 835–844.

Borg, C., Jalil, A., Laderach, D., Maruyama, K., Wakasugi, H., Charrier, S., Ryffel, B., Cambi, A., Figdor, C., Vainchenker, W., Galy, A., Caignard, A., Zitvogel, L., 2004. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. Blood 104, 3267–3275.

Boysen, P., Olsen, I., Berg, I., Kulberg, S., Johansen, G.M., Storset, A.K., 2006a. Bovine CD2−/NKp46+ cells are fully functional natural killer cells with a high activation status. BMC Immunol. 7, 10.

Boysen, P., Klevar, S., Olsen, I., Storset, A.K., 2006b. The protozoan *Neospora caninum* directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. Infect. Immun. 74, 953–960.

Brown, W.C., Norimine, J., Knowles, D.P., Goff, W.L., 2006. Immune control of *Babesia bovis* infection. Vet. Parasitol. 138, 75–87.

Buddle, B.M., Wedlock, D.N., Denis, M., Skinner, M.A., 2005. Identification of immune response correlates for protection against bovine tuberculosis. Vet. Immunol. Immunopathol. 108, 45–51.

de Saint-Vis, B., Fugier-Vivier, I., Massacrier, C., Gaillard, C., Vanbervliet, B., it-Yahia, S., Banchereau, J., Liu, Y.J., Lebecque, S., Caux, C., 1998. The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. J. Immunol. 160, 1666–1676.

Denis, M., Keen, D.L., Parlane, N.A., Storset, A.K., Buddle, B.M., 2007. Bovine natural killer cells restrict the replication of *Mycobacterium bovis* in bovine macrophages and enhance IL-12 release by infected macrophages. Tuberculosis 87, 53–62.

Endsley, J.J., Endsley, M.A., Estes, D.M., 2006. Bovine natural killer cells acquire cytotoxic/effector activity following activation with IL-12/15 and reduce *Mycobacterium bovis* BCG in infected macrophages. J. Leukoc. Biol. 79, 71–79.

Ferlazzo, G., Tsang, M.L., Moretta, L., Melioli, G., Steinman, R.M., Munz, C., 2002. Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. J. Exp. Med. 195, 343–351.

Fernandez, N., Flament, C., Crépineau, F., Angevin, E., Vivier, E., Zitvogel, L., 2002. Dendritic cells (DC) promote natural killer (NK) cell functions: dynamics of the human DC/NK cell cross talk. Eur. Cytokine Netw. 13, 17–27.

Fischer, K., Mackensen, A., 2003. The flow cytometric PKH-26 assay for the determination of T-cell mediated cytotoxic activity. Methods 31, 135–142.

Gerosa, F., Baldani-Guerra, B., Nisii, C., Marchesini, V., Carra, G., Trinchieri, G., 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. J. Exp. Med. 195, 327–333.

Gerosa, F., Gobbi, A., Zorzi, P., Burg, S., Briere, F., Carra, G., Trinchieri, G., 2005. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. J. Immunol. 174, 727–734.

Goff, W.L., Davis, W.C., Palmer, G.H., McElwain, T.F., Johnson, W.C., Bailey, J.F., McGuire, T.C., 1988. Identification of *Babesia bovis* merozoite surface antigens by using immune bovine sera and monoclonal antibodies. Infect. Immun. 56, 2363–2368.

Goff, W.L., Johnson, W.C., Wyatt, C.R., Cluff, C.W., 1996. Assessment of bovine mononuclear phagocytes and neutrophils for induced L-arginine-dependent nitric oxide production. Vet. Immunol. Immunopathol. 55, 45–62.

Goff, W.L., McElwain, T.F., Suarez, C.E., Johnson, W.C., Brown, W.C., Norimine, J., Knowles, D.P., 2003. Competitive enzyme-linked immunosorbent assay based on a rhoptry-associated protein 1 epitope specifically identifies *Babesia bovis*-infected cattle. Clin. Diagn. Lab. Immunol. 10, 38–43.

Goff, W.L., Storset, A.K., Johnson, W.C., Brown, W.C., 2006. Bovine splenic NK cells synthesize IFN-gamma in response to IL-12-containing supernatants from *Babesia bovis*-exposed monocyte cultures. Parasite Immunol. 28, 221–228.

Haase, C., Jørgensen, T.N., Michelsen, B., 2002. Both exogenous and endogenous interleukin-10 affects the maturation of bone-marrow-derived dendritic cells *in vitro* and strongly influences T-cell priming *in vivo*. Immunology 107, 489–499.

Hope, J.C., Thom, M.L., McCormick, P.A., Howard, C.J., 2004. Interaction of antigen presenting cells with mycobacteria. Vet. Immunol. Immunopathol. 100, 187–195.

Howard, C.J., Sopp, P., Brownlie, J., Kwong, L.S., Parsons, K.R., Taylor, G., 1997. Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. J. Immunol. 159, 5372–5382.

Iwamoto, S., Iwai, S.I., Tsujiyama, K., Kurahashi, C., Takeshita, K., Naoe, M., Masunaga, A., Ogawa, Y., Oguchi, K., Miyazaki, A., 2007. TNF-{alpha} drives human CD14+ monocytes to differentiate into CD70+ dendritic cells evoking Th1 and Th17 responses. J. Immunol. 179, 1449–1457.

Klevar, S., Kulberg, S., Boysen, P., Storset, A.K., Moldal, T., Bjorkman, C., Olsen, I., 2007. Natural killer cells act as early responders in an experimental infection with *Neospora caninum* in calves. Int. J. Parasitol. 37, 329–339.

Korbel, D.S., Finney, O.C., Riley, E.M., 2004. Natural killer cells and innate immunity to protozoan pathogens. Int. J. Parasitol. 34, 1517–1528.

Lanier, L.L., 2005. NK cell recognition. Ann. Rev. Immunol. 23, 225–274.

Lucas, M., Schachterle, W., Oberle, K., Aichele, P., Diefenbach, A., 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 26, 503–517.

Medvedev, A.E., Johnsen, A.C., Haux, J., Steinkjer, B., Egeberg, K., Lynch, D.H., Sundan, A., Espevik, T., 1997. Regulation of Fas and Fas-ligand expression in NK cells by cytokines and the involvement of Fas-ligand in NK/LAK cell-mediated cytotoxicity. Cytokine 9, 394–404.

Moretta, A., 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. Nat. Rev. Immunol. 2, 957–964.

Moretta, A., 2005. The dialogue between human natural killer cells and dendritic cells. Curr. Opin. Immunol. 17, 306–311.

Moretta, L., Ferlazzo, G., Mingari, M.C., Melioli, G., Moretta, A., 2003. Human natural killer cell function and their interactions with dendritic cells. Vaccine 21, S38–S42.

Mwangi, W., Brown, W.C., Lewin, H.A., Howard, C.J., Hope, J.C., Baszler, T.V., Caplazi, P., Abbott, J., Palmer, G.H., 2002. DNA-encoded fetal liver tyrosine kinase 3 ligand and granulocyte macrophage-colony-stimulating factor increase dendritic cell recruitment to the inoculation site and enhance antigen-specific CD4+ T cell responses induced by DNA vaccination of outbred animals. J. Immunol. 169, 3837–3846.

Olsen, I., Boysen, P., Kulberg, S., Hope, J.C., Jungersen, G., Storset, A.K., 2005. Bovine NK cells can produce gamma interferon in response to the secreted mycobacterial proteins ESAT-6 and MPP14 but not in response to MPB70. Infect. Immun. 73, 5628–5635.

Oshimi, Y., Oda, S., Honda, Y., Nagata, S., Miyazaki, S., 1996. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. J. Immunol. 157, 2909–2915.

Piccioli, D., Sbrana, S., Melandri, E., Valiante, N.M., 2002. Contact-dependent stimulation and inhibition of dendritic cells by natural killer cells. J. Exp. Med. 195, 335–341.

Sandau, M.M., Schluns, K.S., Lefrancois, L., Jameson, S.C., 2004. Cutting edge: Transpresentation of IL-15 by bone marrow-derived cells necessitates expression of IL-15 and IL-15R{alpha} by the same cells. J. Immunol. 173, 6537–6541.

Scharton-Kersten, T.M., Sher, A., 1997. Role of natural killer cells in innate resistance to protozoan infections. Curr. Opin. Immunol. 9, 44–51.

Shibata, Y., Honda, I., Justice, J.P., Van Scott, M.R., Nakamura, R.M., Myrvik, Q.N., 2001. Th1 adjuvant N-acetyl-d-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. Infect. Immun. 69, 6123–6130.

Storset, A.K., Kulberg, S., Berg, I., Boysen, P., Hope, J.C., Dissen, E., 2004. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur. J. Immunol. 34, 669–676.

Varma, S., Shatry, A.M., 1980. A technique for partial marsupialisation of the spleen in calves. Vet. Rec. 106, 127–128.

Walzer, T., Dalod, M., Robbins, S.H., Zitvogel, L., Vivier, E., 2005. Natural-killer cells and dendritic cells: ''l'union fait la force''. Blood 106, 2252–2258.

Wang, Y.S., Chi, K.H., Chu, R.M., 2007. Cytokine profiles of canine monocyte-derived dendritic cells as a function of lipopolysaccharide- or tumor necrosis factor-alpha-induced maturation. Vet. Immunol. Immunopathol. 118, 186–198.

Yamashita, M., Shinohara, T., Tsuji, S., Myrvik, Q.N., Nishiyama, A., Henriksen, R.A., Shibata, Y., 2007. Catalytically inactive cyclooxygenase 2 and absence of prostaglandin E2 biosynthesis in murine peritoneal macrophages following in vivo phagocytosis of heat-killed *Mycobacterium bovis* Bacillus Calmette-Guerin. J. Immunol. 179, 7072–7078.

Zhuang, Y., Mwangi, W., Brown, W.C., Davis, W.C., Hope, J.C., Palmer, G.H., 2006. Characterization of a phenotypically unique population of CD13+ dendritic cells resident in the spleen. Clin. Vacc. Immunol. 13, 1064–1069.